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13. ABSTRACT ***(Maximum 200 Words)***

Here we report the results of our pilot studies to find new approaches to fight the propensity of breast cancer cells to metastasize. Since the latter depends on increased motility secondary to degradation of extracellular matrix (ECM) we studied the inhibitors of matrix metalloproteinases (MMPs). MMPs activate proteolysis of ECM and are implicated in cancer and metastasis. As a model of cell motility we used *Xenopus laevis* embryos because their germ cell layers undergo extensive movements in the gastrula stage of development. Injection of a synthetic mRNA encoding an inhibitor of MMPs, Xtimp3, led to shortening of the embryonic anterior-posterior axis and to eye defects, indicating defect of motility. We concluded that Xtimp3 interferes with cell motility. Similar movement defects are produced by Wnt oncogen receptors of Frizzled family. We tested for the involvement of a Frizzled receptor, Xfz8, in carcinogenesis and found that Xfs8 triggered apoptosis in gastrulating *Xenopus* embryos. Induction of apoptosis by Xfz8 required the cytoplasmic tail of the receptor (Appendix, Figure 3) and depended on the activation of c-jun N-terminal kinases. It occurred in ß-catenin and convergent extension pathway-independent manner. These results raise the possibility that Wnt/Frizzled signaling may control tumor growth by regulating apoptosis in cancer cells.

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Introduction

Metastases are the cause of morbidity and death in breast cancer patients. Breast cancer tumors have a strong tendency to metastasize in bone. It is believed that breast cancer cells are recruited to the bone tissue due to several factors secreted by osteoblasts (1). Indeed, one of them, osteonectin, has been shown to elicit positive chemotaxis in breast and prostate cancer cell lines (2). The high motility and invasiveness of cancer cells depend on their altered adhesive properties and the ability to degrade the extracellular matrix (ECM, 3). In order to arrest such cells in situ, their attachment to the surrounding cells and ECM should be increased and their ECM degrading activity should be blocked. We suggested a novel approach, whereby instead of being fought against, cancer cells with the potential for metastasis are attracted to a spot in the tissue of the primary tumor and consequently entrapped. Factors altering cell adhesiveness and cell ability to degrade ECM can be used along with the chemoattractants to design a 'metastasis trap'.

Body

Since the invasiveness of cancer cells is suspected to depend on the ability to degrade extracellular matrix (ECM) to become motile we focused on studying matrix metalloproteinases (MMPs), which are enzymes controlling proteolytic degradation of the ECM in cells and tissues and are implicated in cancer and metastasis (4). To establish an assay in which we could test the importance of extracellular matrix for cell motility we used an embryological model. Many MMPs are expressed only during embryogenesis suggesting that the degradation and remodeling of ECM play an important role in development. *Xenopus laevis* embryos provide a convenient system for observing cell motility because germ cell layers of the embryo undergo extensive movements in the gastrula stage of development. Defects of these movements are easily observable due to resulting structural embryonic abnormalities. To inhibit MMP activity during gastrulation, we used overexpression of a *Xenopus* tissue inhibitor of metalloproteinases-3 (Xtimp3, 5). Synthetic mRNA encoding Xtimp3 was micro-injected into 4-cell stage *Xenopus* embryos. This led to the shortening of the anterior-posterior axis of developing embryos and to eye defects, the typical phenotypes produced by interfering with the morphogenetic movements of gastrulation (Fig. 1). We concluded from this experiment that the inhibitor of metalloproteinases Xtimp3 interferes with cell motility. We also observed that the similar movement defects are produced by some Frizzled transmembrane receptors of Wnt oncogens, which are implicated in the development of several tumors including breast carcinomas (6). Since there has not been much progress on the analysis of Timp3 effects in cancer cell lines, we decided to test how Frizzled signaling may be involved in carcinogenesis. We found that a Frizzled receptor, Xfz8, triggered rapid cell death in gastrulating *Xenopus* embryos upon overexpression (Appendix, Figure 1), suggesting that Frizzled signaling leads to apoptosis. Since tumor cell growth is associated with the decreased propensity of cells to apoptose these experiments also suggested that Frizzled signaling may be important in the control of tumor cell growth.. Therefore, we thought that activation of apoptosis by Frizzled receptors may represent a new approach to the control of cancer cell proliferation. To explore this possibility further we next measured caspase activation and DNA fragmentation and proved that the cell death induced by Xfz8 is due to apoptosis

(Appendix, Figure 2). Induction of apoptosis by Xfz8 required the cytoplasmic tail of the receptor (Appendix, Figure 3) and correlated with the activation of c-jun N-terminal kinases (JNK, Appendix, Figure 4A) suggesting that JNK is involved in signaling to apoptosis. A dominant negative form of an upstream kinase SEK1, a specific inhibitor of JNK, blocked JNK activation (Appendix, Figure 4D) arguing for the involvement of JNK in Xfz8-dependent apoptosis. The apoptotic signaling was shared by a specific subset of Frizzled receptors (Appendix, Figure 5E), was inhibited by Wnt5A (Appendix, Figure 5B) and occurred in ß-catenin and convergent extension pathway-independent manner (Appendix, Figure 5C). Thus, these experiments identify a new Frizzled-dependent signaling pathway, which involves JNK and can lead to apoptosis. These results raise the possibility that Wnt/Frizzled signaling may regulate apoptosis in cancer cells.

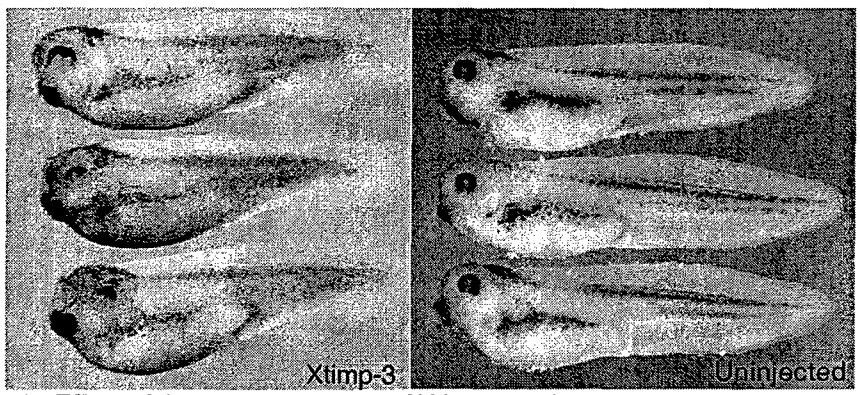

Figure 1. Effect of the overexpression of Xtimp3 on the developing *Xenopus* embryos. Axial structures are shortened, eyes have defects. Xtimp3 RNA was injected into dorsal vegetal blastomeres at the 4-cell stage.

Key Research Accomplishments

- An inhibitor of metalloproteinases Xtimp3 inhibits cellular motility.
- A Frizzled receptor of Wnt oncogen, Xfz8, can induce apoptosis.
- Frizzled-dependent signaling to apoptosis requires c-Jun N-terminal kinases.

Reportable Outcomes

- M. Lisovsky, K. Itoh and S. Sokol: Frizzled receptors activate a novel JNK-dependent Pathway that may lead to Apoptosis. *Curr Biol*, 12, 53-58, 2002
- M. Lisovsky, K. Itoh and S. Sokol: *Xenopus* Frizzled 8 activate apoptosis. Oral Presentation. International Wnt meeting, New York, 2001.

Conclusions

Our findings show that manipulation of extacellular matrix by Xtimp3 affects cell motility and thus has a potential to restrict cancer cell metastases. It also shows that the transmembrane Frizzled receptors may control cancer cell proliferation by activation of JNK-dependent apoptosis.

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Appendices

7. Original copy of: M. Lisovsky, K. Itoh and S. Sokol: Frizzled receptors activate a novel JNK-dependent Pathway that may lead to Apoptosis. *Curr Biol*, 12, 53-58, 2002.

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Frizzled Receptors Activate a Novel JNK-Dependent Pathway that May Lead to Apoptosis

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Summary

Extracellular Wnt ligands and their receptors of the Frizzled family control cell fate, proliferation, and polarity during metazoan development. Frizzled signaling modulates target gene expression through a β-catenin-dependent pathway, functions to establish planar cell polarity in *Drosophila* epithelia, and activates convergent extension movements and intracellular Ca^{2+} signaling in frog and fish embryos [1–6]. Here, we report that a Frizzled receptor, *Xenopus* Frizzled 8 (Xfz8) [7, 8], activates c-Jun N-terminal kinases (JNK) and triggers rapid apoptotic cell death in gastrulating *Xenopus* embryos. This activity of Xfz8 required the cytoplasmic tail of the receptor and was blocked by a dominant inhibitor of JNK. Moreover, the cytoplasmic tail of Xfz8 targeted to the membrane was sufficient for activation of JNK and apoptosis. The apoptotic signaling was shared by a specific subset of Frizzled receptors, was inhibited by Wnt5a, and occurred in a Dishevelled- and T cell factor (TCF)-independent manner. Thus, our experiments identify a novel Frizzled-dependent signaling pathway, which involves JNK and differs from the β-catenin-dependent and convergent extension pathways.

Results and Discussion

In the canonical Wnt pathway, Frizzled receptors signal to the Dishevelled (Dsh) protein, which causes accumulation of cytoplasmic β-catenin. After translocation to the nucleus, β-catenin activates T cell factor (TCF)-dependent target genes [1, 2]. Another signaling pathway that involves Frizzled and Dsh is the planar cell polarity pathway (PCP) that controls directional hair growth in *Drosophila* eye, wing, and leg epidermis [5, 6]. Among the molecular components of this pathway are small GTPases of the Rho family and c-Jun N-terminal kinase (JNK) [6]. In vertebrates, a pathway related to PCP regulates convergent extension movements [9–11], which involve mediolateral polarization and intercalation of cells and are essential for body axis elongation during gastrulation and neurulation [12]. Besides signaling through the β-catenin and PCP pathways, some Frizzled receptors are able to stimulate G protein-dependent intracellular Ca^{2+} release and activate protein kinase C (PKC) and calmodulin-dependent protein kinase II [4, 13].

Xfz8 activates β-catenin-mediated signal transduction [7, 8] and participates in the control of convergent extension movements [8, 14]. To search for additional phenotypic consequences of Xfz8 signaling, 5 ng Xfz8 mRNA was microinjected into the animal region of all blastomeres of the four-cell embryos. Injected embryos developed without visible abnormalities until gastrulation. At midgastrula stages, a white mottled area became apparent in the animal hemisphere. The majority of injected embryos (113 out of 122) disintegrated and died by neurula stages (Figure 1A). Injection of a single blastomere, or injection of lower doses of Xfz8 RNA (2.5 ng per blastomere), caused the same morphological phenotype in a more limited area of the embryo (data not shown). Embryos injected with 5 ng Xfz3 RNA encoding a different Frizzled homolog (Figure 1A) [15], or C-terminally truncated Xfz8, lacking the last 17 amino acids (ΔC-Xfz8, Figures 1A and 1B), developed normally, attesting to the specificity of the Xfz8 effect.

To assess whether observed morphological abnormalities were due to apoptosis [16–18], we first stained the injected embryos with Sytox, a DNA-intercalating dye impermeable to live cells. Morphologically abnormal areas in embryos expressing Xfz8 contained bright fluorescent cells, indicating that Xfz8 induced cell death (Figure 2A). We next analyzed DNA fragmentation and activation of caspase-3-like proteases, two key markers of apoptosis [19, 20]. DNA from embryos overexpressing Xfz8 showed characteristic internucleosomal fragmentation (Figure 2B). Also, Xfz8 strongly upregulated caspase-3-related activity (Figure 2C). This effect was dose dependent (Figure 2D) and was suppressed by the broad-range caspase inhibitor Boc-D-fmk added to the reaction mixture (Figure 2C). Neither ΔC-Xfz8 nor Xfz3 caused DNA fragmentation or caspase activation (Figures 2B and 2C), yet Xfz8 and ΔC-Xfz8 were equally potent in their ability to induce a secondary axis, and Xfz3 interfered with convergent extension to the same degree as Xfz8, indicating similar biological activity (data not shown) [7, 14]. These results suggest that induction of apoptosis by Xfz8 is specific and requires the presence of the C-terminal sequences.

Despite the high degree of conservation of the cytoplasmic tails among individual Frizzled orthologs, no function has been previously demonstrated for the C-terminal portion of the cytoplasmic tail. Our findings present the first evidence that the C-terminal part of the cytoplasmic tail is involved in Frizzled signaling. To further define structural requirements for apoptotic signaling by Xfz8, we asked whether the cytoplasmic tail of Xfz8 was sufficient for induction of apoptosis. A myristoylation signal was attached to the cytoplasmic tail of Xfz8 (myr-CT, Figure 1B) to target it to the cell membrane [21]. Myr-CT activated caspase-3-like proteases and caused cell death in a manner similar to Xfz8 (Figure 3A and data not shown). In contrast, ΔC-myr-CT lacking the C-terminal 17 amino acids and GST-CT, a fusion of

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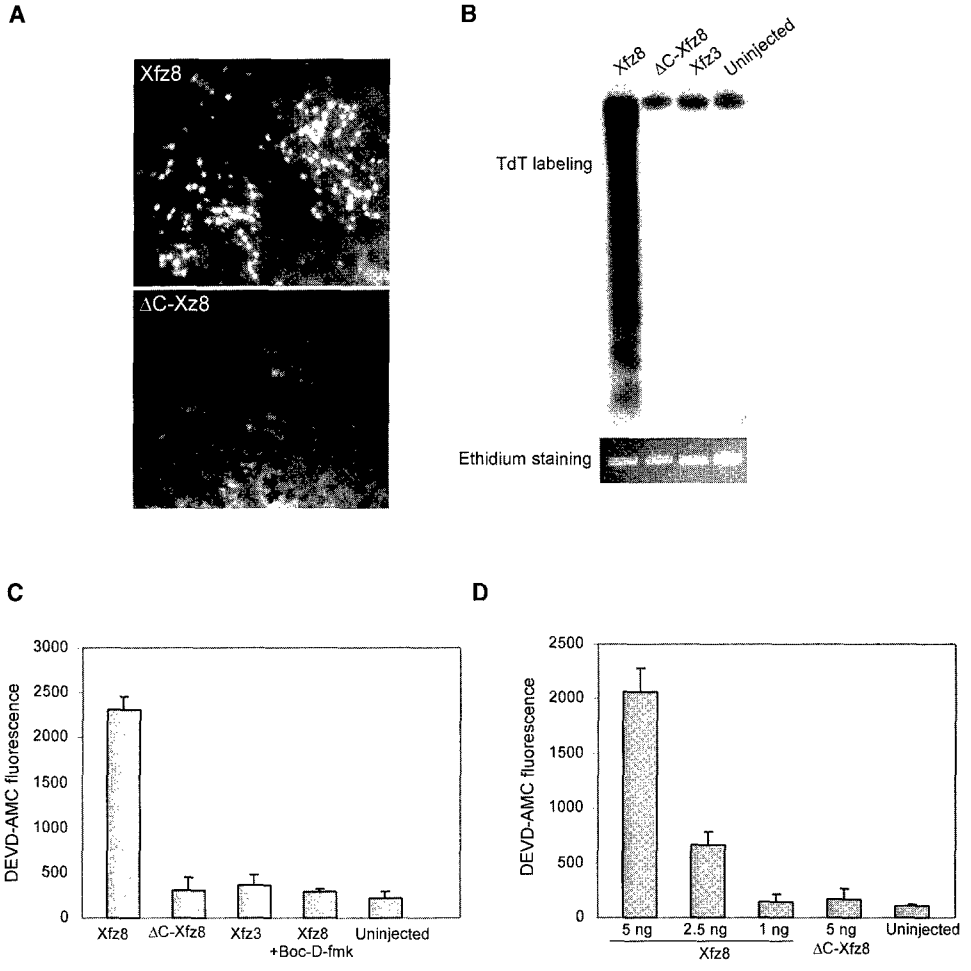

Figure 2. Apoptosis Induced by Xfz8

(A) Sytox-positive cell staining in embryos injected with Xfz8, but not with ΔC-Xfz8 RNA.

(B) Internucleosomal DNA fragmentation triggered by Xfz8. Separation of ^{32}P-end-labeled genomic DNA on a 2% agarose gel shows a characteristic fragmentation ladder for embryos overexpressing Xfz8. Ethidium bromide staining reflects loading.

(C) Caspase activation by Xfz8. Xfz8-dependent caspase activity is suppressed by the addition of the broad-range caspase inhibitor Boc-D-fmk to the reaction mixture (0.2 mM final concentration).

(D) The effect of Xfz8 on caspase activity is dose dependent. (C and D) Activation of caspase-3-related proteases was measured in triplicate by the amount of cleaved fluorogenic substrate DEVD-AMC as described in the Supplementary Material. Means ± standard errors of a representative experiment are shown. (A–D) Four-cell embryos were injected into each blastomere with 5 ng RNA as indicated and were cultured until stage 13. Each experiment has been repeated at least three times.

Xwnt5A and ΔN-Xfz8. Contrary to our expectations, Xwnt5A prevented caspase activation by ΔN-Xfz8 (Figure 5B), suggesting that the inhibitory effect of Xwnt5A is not mediated through the ligand binding domain of Xfz8.

To assess whether signaling to apoptosis requires previously characterized β-catenin-mediated and convergent extension pathways, we used several specific inhibitors. Dominant-negative forms of the *Xenopus* Dsh (Xdd1) and Tcf3 (ΔN-Xtcf3) have been shown to inhibit β-catenin signaling [9, 27]. Also, Xdd1 and another dominant-negative form of *Xenopus* Dsh, Dsh-DEP+ [10], were reported to suppress convergent extension movements [9, 11]. Neither of these constructs suppressed Xfz8-mediated caspase activation (Figure 5C); although, at the same dose, both Xdd1 and Dsh-DEP+ strongly inhibited convergent extension movements (data not shown). Also, ΔN-Xtcf3 RNA significantly decreased the frequency of axis duplication induced by Xwnt8 RNA from 62% to 18% of injected embryos (n = 26 and 29, respectively). These results suggest that the β-catenin-dependent and convergent extension pathways are not involved in Xfz8-mediated apoptosis.

To determine whether other Frizzled homologs share with Xfz8 the ability to induce apoptosis, embryos were injected with RNAs encoding rat Fz1 (rFz1), rat Frizzled 2 (rFz2), human Fz5 (hFz5), and *Xenopus* Fz7 (Xfz7). Xfz7, rFz1, and hFz5 function through the β-catenin and convergent extension pathways [3, 28–30]. RFz2 stimulates Ca^{2+} release and activates PKC [4, 13]. HFz5, but not other tested Frizzled receptors, induced morphological abnormalities similar to those caused by Xfz8 (Figure 5D, 51 out of 56 injected embryos) and activated caspase 3-like proteases (Figure 5E). Biological activity of

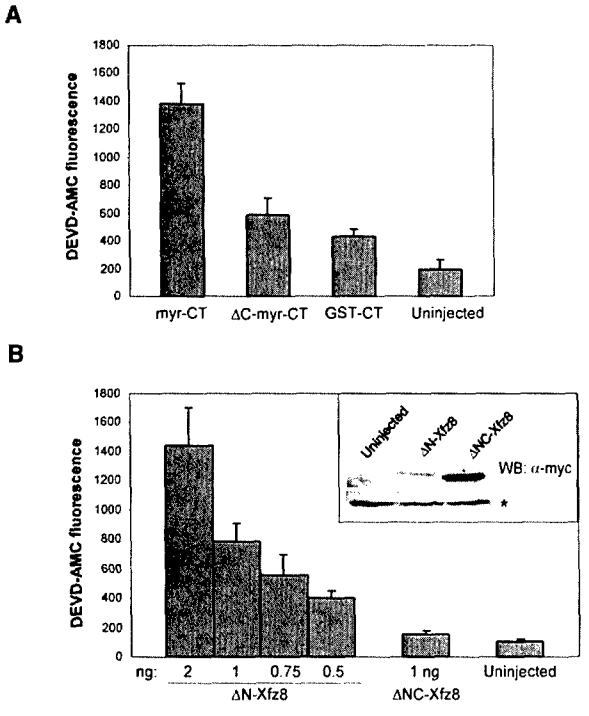

Figure 3. A Role for the Cytoplasmic Tail of Xfz8 in Caspase Activation

(A) A myristoylated form of the cytoplasmic tail of Xfz8 (myr-CT), but not ΔC-myr-CT or GST-CT, activates caspase-3-like proteases. Four-cell embryos were injected with 1 ng of the indicated RNAs.
(B) Dose-dependent activation of caspases by ΔN-Xfz8. Indicated amounts of ΔN-Xfz8 RNA and 1 ng ΔNC-Xfz8 RNA were injected. The inset shows expression levels of ΔN-Xfz8 and ΔNC-Xfz8 detected in embryo lysates with anti-myc antibody. An asterisk indicates a nonspecific protein band reflecting protein loading. (A and B) Embryos were cultured until stages 11.5–12.

rFz1 and Xfz7 RNAs was verified by their ability to alter convergent extension movements, and the activity of rFz2 RNA was confirmed by the recruitment of PKC to the cell membrane (data not shown). These data show that the apoptosis-inducing activity does not correlate with other known signaling properties of Frizzled receptors. Interestingly, the C-terminal region of the cytoplasmic tail of hFz5, but not the other tested Frizzled receptors, shares significant similarity with the corresponding region of Xfz8, required for JNK activation and apoptosis. Taken together, these results are consistent with the view that signaling to apoptosis is distinct from previously described Fizzled-signaling pathways.

In our experiments, cell death triggered by Xfz8 was observed only after the onset of gastrulation, consistent with findings that the apoptotic program is suppressed at earlier stages of development [16–18]. Apoptosis was induced at relatively high doses of Xfz8 RNA (2.5–5 ng), whereas 1–2 ng has been sufficient for activation of the β-catenin and convergent extension pathways [7]. This disparity may be due to stronger stimulation of an already known pathway or to activation of a novel signaling pathway. Several lines of evidence argue in favor of the latter possibility. First, Xwnt5A and Xwnt8 failed to cooperate with Xfz8 in apoptotic signaling, whereas the same Wnts synergize with Xfz8 in the β-catenin-dependent axis induction assay [7, 8]. Second, Xfz3, Xfz7, and rFz1, which activate β-catenin or convergent extension signaling [3, 15, 28, 31], could not trigger apoptosis. Third, dominant interfering forms of Dsh and Tcf3, which block the β-catenin and convergent extension pathways, did not suppress apoptosis triggered by Xfz8. Fourth, although rFz2 was reported to stimulate Ca^{2+} release [4, 13], it failed to trigger cell death in *Xenopus* embryos. Conversely, hFz5 induced apoptosis, but was reported not to activate PKC [13]. Finally, the structural requirements for the apoptotic pathway are quite different. ΔC-Xfz8 stimulates β-catenin-dependent signaling [7], yet it fails to trigger apoptosis. Together, these observations indicate that Frizzled receptors activate JNK and apoptosis through a novel pathway. Future studies are required to further define the significance of this pathway in embryogenesis and to assess whether activation of apoptosis reflects an endogenous role of Frizzled signaling in the embryo.

Supplementary Material

Supplementary Material including the Experimental Procedures is available at http://images.cellpress.com/supmat/supmatin.htm.

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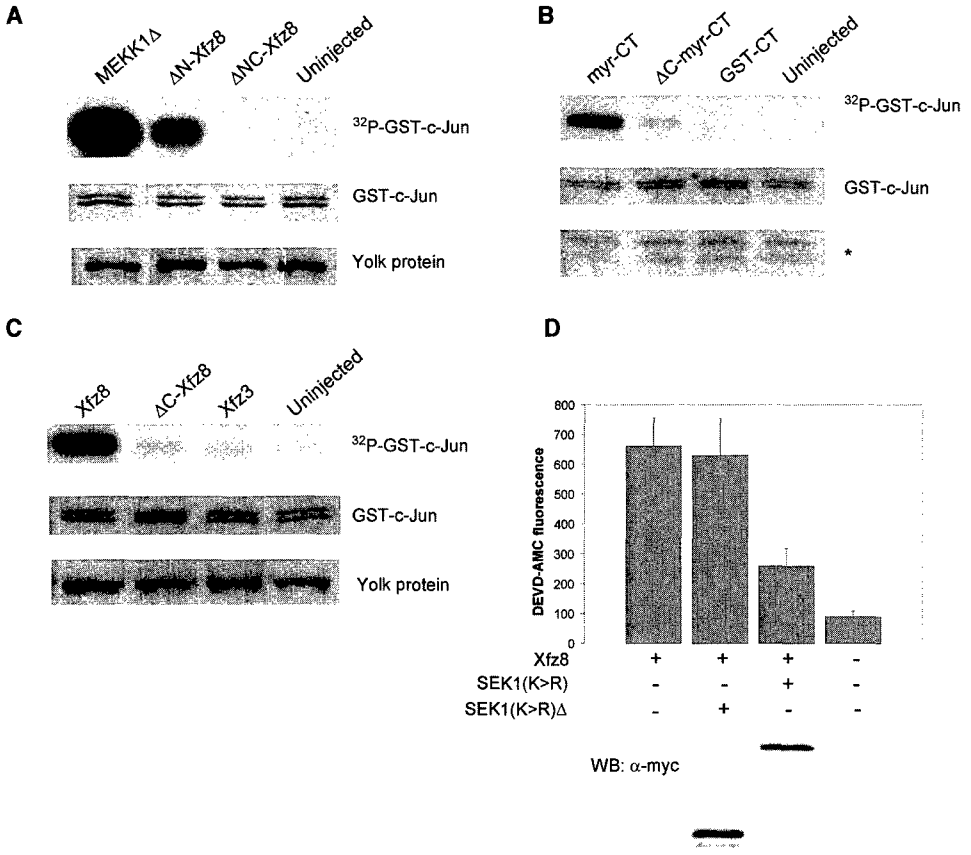

Figure 4. Induction of Apoptosis by Xfz8 Involves c-Jun N-Terminal Kinase

(A–C) Activation of JNK by Xfz8 constructs is revealed in embryonic lysates by in vitro phosphorylation of GST-c-Jun(1–135). JNK was strongly induced by (A) ΔN-Xfz8, (B) myr-CT, and (C) Xfz8, but not by ΔNC-Xfz8, ΔC-myr-CT, GST-CT, ΔC-Xfz8, or Xfz3 RNAs. (A) A constitutively active MEKK1, MEKK1Δ, served as a positive control. Coomassie-stained gels show equal loading of GST-c-Jun(1–135). Protein loading is reflected by two nonspecific bands indicated by an asterisk (B) and the 100-kDa band corresponding to a yolk protein (A and C).

(D) SEK1(K>R) prevents caspase activation by Xfz8. Expression levels of SEK1(K>R) and SEK1(K>R)Δ were assessed using anti-myc antibody and are shown at the bottom of (D). RNAs were injected as described in Figure 1. The following amounts of RNA were injected: ΔN-Xfz8, 1 ng; ΔNC-Xfz8, 1 ng; myr-CT, 1 ng; ΔC-myr-CT, 1 ng; Xfz8, 5 ng; ΔC-Xfz8, 5 ng; Xfz3, 5 ng; SEK1(K>R), 5 pg; SEK1(K>R)Δ, 400 pg. Embryos were collected for analysis at stages (A and B) 11.5–12 and at (C and D) stage 13.

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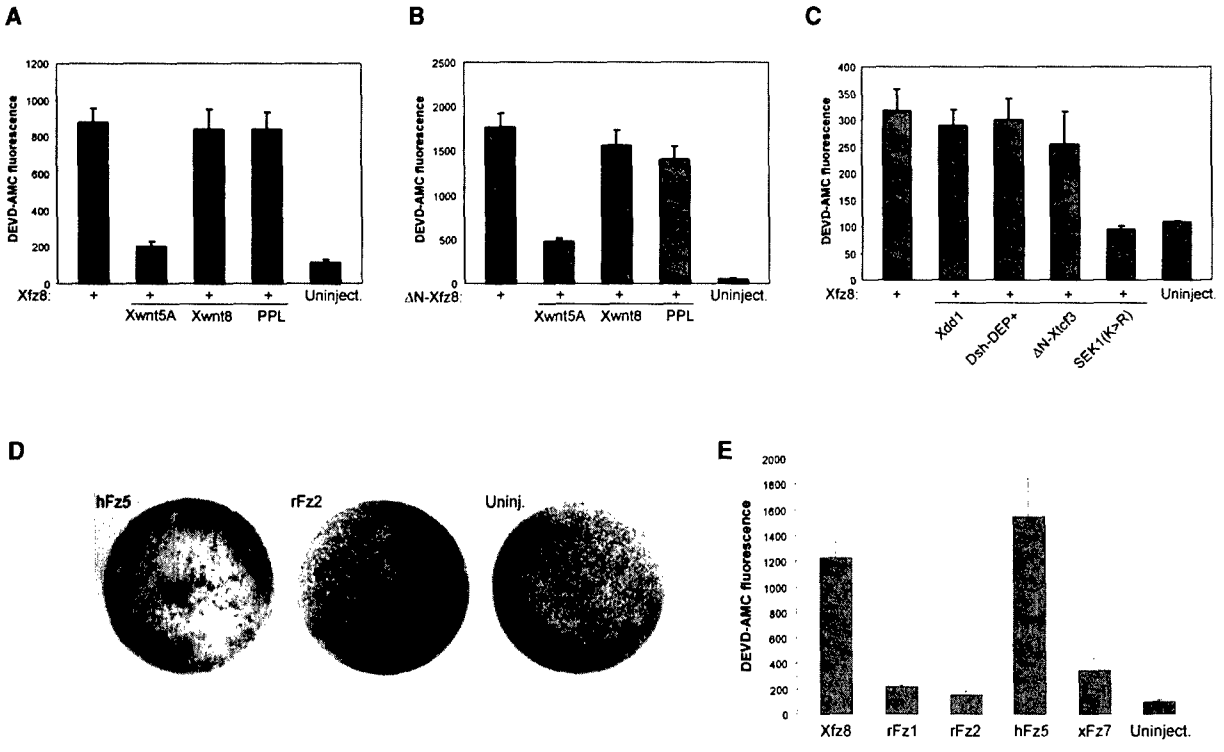

Figure 5. Apoptosis Triggered by Xfz8 Reflects a Novel Signaling Pathway

(A and B) Xwnt5A suppresses caspase activation by Xfz8 or ΔN-Xfz8. Four-cell embryos were injected into the animal hemisphere with (A) 5 ng Xfz8 RNA or (B) 1 ng ΔN-Xfz8 RNA, alone or together with Xwnt5A, Xwnt8, or control preprolactin (PPL) RNAs (0.25 ng each), as indicated.

(C) The apoptotic pathway does not require Dsh and Tcf3 functions. RNAs were injected in the following amounts: Xfz8, 5 ng; Xdd1, 0.5 ng; Dsh-DEP+, 0.5 ng; ΔN-Xtcf3, 60 pg; and SEK1(K>R), 0.3 ng.

(D) Morphological abnormalities induced by hFz5 RNA at stage 12.

(E) Caspase-3-related activity in embryos injected with Xfz8, rFz1, rFz2, hFz5, and Xfz7 RNAs. (D and E) Each RNA was injected at 5 ng. Caspase activity was measured as in Figure 2.

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